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(54) Title: IMPROVED SOLID-PHASE PEPTIDE SYNTHESIS AND AGENT FOR USE IN SUCH SYNTHESIS

(57) Abstract

Peptides X-AA_1-AA_2...AA_n-Y, wherein AA is an L- or D-amino acid residue, X is hydrogen or an amino protective group, Y is OH, NH_2 or an amino acid sequence comprising from 3 to 9 amino acid residues and n is an integer greater than 2, are prepared by solid phase synthesis, preferably using Fmoc-chemistry, the improvement consisting in that the C-terminal part attached to the solid phase comprises a pre-sequence comprising from 3 to 9, preferably from 5 to 7, amino acid residues independently selected from native L-amino acids having a side chain functionality which is suitably protected during the coupling steps and having a propensity factor $P\alpha > 0.57$ and a propensity factor $P\beta > 1.10$, preferably Lys and/or Glu, or the corresponding D-amino acids and said pre-sequence is optionally cleaved from the formed peptide.

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Improved solid-phase peptide synthesis and agent for use in such synthesis.

Background of the invention

1. Technical Field

The present invention relates to an improved process for the production of peptides by solid-phase synthesis. The invention also relates to an agent which is useful in solid-phase peptide synthesis.

2. Background Art

Solid-phase peptide synthesis (SPPS) is a highly successful method introduced by Merrifield in 1963 (Ref. 1). Numerous peptides have been synthesized with this technique since then. An excellent review of the current chemical synthesis of peptides and proteins is provided by S.B.H. Kent (Ref. 2) which is incorporated herein by reference.

In practice, two strategies for the assembly of peptide chains by solid-phase synthesis are used, viz. the stepwise solid-phase synthesis, and solid-phase fragment condensation.

In stepwise SPPS, the C-terminal amino acid in the form of an N-α-protected, if necessary side-chain protected reactive derivative is covalently coupled either directly or by means of a suitable linker to a "solid" support, e.g. a polymeric resin, which is swollen in an organic solvent. The N-α-protective group is removed, and the subsequent protected amino acids are added in a stepwise fashion.

When the desired peptide chain length has been obtained, the side-chain protective groups are removed,

and the peptide is cleaved from the resin, which might be done in separate steps or at the same time.

In solid-phase fragment condensation the target sequence is assembled by consecutive condensation of fragments on a solid support using protected fragments prepared by stepwise SPPS.

Over the years, two coupling strategies have been developed based on the use of different N-α-protective groups and matching side-chain protective groups.

Merrifield used tert.butyloxycarbonyl (Boc) as the N-α protective group, while 9-fluorenylmethyloxycarbonyl (Fmoc) was introduced by Carpino and Han (Ref. 12).

The operations involved in one cycle of chain extension in stepwise SPPC using Boc- and Fmoc-chemistries are illustrated in the reaction-schemes below (taken from Ref. 2).

A.
Boc-AA$_n$-OBzlCH$_2$CONH-CH$_2$-RESIN
Deprotect
Tfa-O$^-$ $^+$NH$_3$-AA$_n$-OBzlCH$_2$CONH-CH$_2$-RESIN
Neutralize
NH$_2$-AA$_n$-OBzlCH$_2$CONH-CH$_2$-RESIN
Couple
(BocAA)$_2$O
BocAA-OH
Boc-AA$_{n-1}$-AA$_n$-OBzlCH$_2$CONH-CH$_2$-RESIN
B.
Fmoc-AA$_n$-O-Bzl-O-CH$_2$-RESIN
Deprotect
NH$_2$-AA$_n$-O-Bzl-O-CH$_2$-RESIN
Couple
FmocAA-OBt
HOBt
Fmoc-AA$_{n-1}$-AA$_n$-O-Bzl-O-CH$_2$-RESIN

The N-α-Boc-protected peptide coupled to a PAM-resin is N-α-deprotected with trifluoro-acetic acid (TFA). The resulting amine salt is washed and neutralized with a tertiary amine. The subsequent peptide bond is formed by reaction with an activated Boc-amino acid, e.g. a symmetric anhydride. Generally, the side-chain protection is benzyl-based, and the deprotection is made with HF or a sulphonic acid.

The N-α-Fmoc protected peptide coupled to a resin is N-α-deprotected by treatment with a secondary amine, normally piperidine, in an organic solvent, e.g. N,N-dimethyl formamide (DMF) or dichloromethane (DCM). After washing, the neutral peptide resin is reacted with an activated Fmoc-amino acid, e.g. a hydroxy-benzotriazole active ester.

The side-chain protection is tert.butyl, trityl and arylsulfonyl based, and for deprotection of the side-chains, preferably TFA is used.

While the Boc- and Fmoc-strategies are used for essentially all current practical peptide synthesis, other N-α protective groups have been proposed (Steward & Young, Ref .13).

Boc forms an acid-labile urethane group, and other proposals of this category are biphenylisopropyloxy-carbonyl (Bpoc), 3,5-dimethoxyphenylisopropyloxy-carbonyl (Ddz), phenylisopropyloxycarbonyl (Poc) and 2,3,5-tetramethylbenzyloxycarboxyl (Tmz).

Other types of N-α protecting groups available include nitrophenylsulfenyl (Nps) which can be removed by either very dilute anhydrous acid, e.g. HCl, or by nucleophilic attack, e.g. with methyl-3-nitro-4-

mercapto benzoate. Also dithiasuccinyl (Dts), which is removable by nucleophilic attack, might be used.

SPPS has the general advantage that it lends itself to fully automated or semi-automated chain assembly chemistry. A system for SPPS under low pressure continuous flow conditions was developed by Dryland & Sheppard (Ref. 7) and was further refined, see Cameron, Meldal & Sheppard (Ref. 14) Holm & Meldal (Ref.15), and WO 90/02605.

While SPPS has now developed to be a cornerstone in protein and peptide synthesis, certain problems still remain to be solved. Since some of these problems might well be related to the peptide structure, a brief discussion is deemed proper.

Empirical predictions of protein conformations have been made by Chou & Fasman (Ref. 6). It is well-known that protein architectures may be described in terms of primary, secondary, tertiary and quaternary structure. The primary structure refers to the amino acid sequence of the protein. The secondary structure is the local spatial organization of the polymer backbone without consideration of the side-chain conformation. As examples of secondary structures, α-helixes, β-sheets and β-turns, which are chain reversal regions consisting of tetrapeptides can be mentioned. The tertiary structure is the arrangement of all the atoms in space, including disulphide bridges and side-chain positions, so that all short- and long-range interactions are considered.

The term quaternary structure may be used to denote the interaction between subunits of the protein, e.g. the α- and β-chains of hemoglobins.

Following a discussion of earlier attempts to correlate protein secondary structure with amino acid compositions, where e.g. Ser, Thr, Val, Ile and Cys were classified as "helix breakers" and Ala, Leu and Glu as "helix formers", while hydrophobic residues were classified as strong "β-formers", and proline together with charged amino acid residues as "β-breakers", Chou & Fasman made a statistical analysis of 29 proteins with known X-ray structure in order to establish prediction rules for α- and β-regions.

Based on these studies they determined so-called propensity factors Pα, Pβ and Pt which are conformational parameters expressing the positional preferences as α-helix, β-sheet and β-turn, respectively, for the natural L-amino acids forming part of proteins.

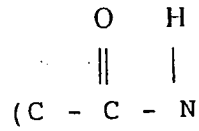
For the sake of convenience, the Pα and Pβ are listed below. One-letter abbreviations for the individual amino acids are given in parenthesis.

	P_α		P_β
Glu (E)	1.51	Val	1.70
Met (M)	1.45	Ile	1.60
Ala (A)	1.42	Tyr	1.47
Leu (L)	1.21	Phe	1.38
Lys (K)	1.16	Trp	1.37
Phe (F)	1.13	Leu	1.30
Gln (Q)	1.11	Cyr	1.19
Trp (W)	1.08	Thr	1.19
Ile (I)	1.08	Gln	1.10
Val (V)	1.06	Met	1.05
Asp (D)	1.01	Arg	0.93
His (H)	1.00	Asn	0.89
Arg (R)	0.98	His	0.87
Thr (T)	0.83	Ala	0.83
Ser (S)	0.77	Ser	0.75
Cys (C)	0.70	Gly	0.75
Tyr (Y)	0.69	Lys	0.74
Asn (N)	0.67	Pro	0.55
Pro (P)	0.57	Asp	0.54
Gly (G)	0.57	Glu	0.37

Generally speaking, values below 1.00 indicate that the amino acid in question must be regarded as unfavourable for the particular secondary structure.

As an example, the hydrophobic acids (e.g. Val, Ile, Leu) are strong β-sheet formers, while the charged amino acids (e.g. Glu, Asp, His) are β-sheet breakers.

In the α-helix structure, the spiral configuration of the peptide is held rigidly in place by hydrogen bonds between the hydrogen atom attached to the nitrogen atom in one repeating unit



and the oxygen atom attached to a carbon atom three units along the chain.

If a polypeptide is brought into solution, the α-helix can be made to unwind to form a random coil, by adjustment of the pH. The transition from α-helix to random coil occurs within a narrow pH. Since the hydrogen bonds are all equivalent in bond strength in the α-helix, they tend to let go all at once. The change can also be induced by heat.

The β-sheet structure consists of fully extended peptide chains in which hydrogen bonds link the hydrogen atoms on one chain to the oxygen atoms in the adjoining chain. Thus hydrogen bonds do not contribute to the internal organization of the chain as they do in the α-helix, but only bond chain to chain. Adjacent chains may be parallel or antiparallel.

β-turns are frequently observed in these parts of a peptide chain which connect antiparallel chains in a β-sheet structure.

In a β-turn, the CO- and NH-groups from amino acid No. n in the peptide chain form hydrogen bond to the corresponding groups in amino acid No. n+4.

α-helix and β-sheet constitute strongly variating parts of the peptide conformation of proteins (from 0 to 80 %), and the remaining parts of the proteins are folded in other structures. In most proteins sections of the peptide chains appear as irregularly folded "random coils".

Turning now to the general problems still prevailing in connection with SPPS, S.B.H. Kent (Ref. 2) highlights the synthesis of "difficult sequences".

Obviously, the whole rationale of SPPS is based on a complete N-α-deprotection prior to each of the coupling steps involved.

By the same token, ideally all of the N-α-deprotected amino groups should be coupled to the reactive amino acid derivative according to the desired sequence, i.e. a complete aminoacylation should take place.

Kent states that the most serious potential problem in stepwise SPPS is incomplete peptide bond formation giving rise to peptides with one or more amino acids missing (deletions), but with properties similar to the target sequence.

Such incomplete couplings are more prevalent in some sequences than in others, hence the term "difficult sequences", and are apparently also more predominant in Fmoc-chemistry than in Boc-chemistry.

A number of recognized "difficult sequences" have been studied by scientists including the present inventors.

During SPPS of homo-oligopeptides containing leucine or alanine using the Fmoc-strategy, ineffective N-α-deprotection with piperidine in a sequence dependent manner (Refs. 3 and 4) was observed. Investigations showed that this phenomenon was associated with subsequent slow or incomplete amino acid coupling and evidence for β-sheet aggregation of the growing peptide chain was presented as a cause for the difficult couplings and incomplete Fmoc-deprotections. This evidence was based on general physical-chemical observations (Ref. 4) and on a detailed Raman Near Infrared Spectroscopic study (Ref. 5). The physical-chemical observations referred to may be summarized as follows: In case of synthesis of H-(Ala)n-Lys-OH on a polyamide polymerized kieselguhr matrix (PepSyn K), no problems were observed until n = 5, but approximately 20 - 25% Fmoc-protected peptide was still present after standard deprotection with piperidine (20% piperidine in DMF) with n = 6. When continuing the synthesis to n = 10 a relatively complicated mixture was obtained comprising the target peptide (n = 10) as well as deletion peptides corresponding to n = 6, 7, 8, and 9 and deletion peptides with the Fmoc group still attached to the N-terminal where n = 6,7,8, and 9 respectively (Fig. 1). This mixture was identified by FAB MS after hplc separation of the single components. Failure sequences or partial deprotection with n = 2 - 5 or incomplete deprotection of the target peptide (n = 10) was not observed, thus confining the problems to a given stretch of the homo-oligopeptide chain. This type of difficult aminoacylations and incomplete deprotections can there-

fore be referred to non-random in contrast to random difficulties which are due only to common steric problems (Ref. 2). Although experimental conditions were varied, e.g. resin-type, deprotection time, solvents, addition of chaotropes, problems still persisted although heating to 50 °C had an optimal effect on incomplete Fmoc-deprotection (Ref. 4).

It is a most characteristic feature of the homo-oligo alanine chain, that the incomplete Fmoc-deprotection steps are followed by strikingly slow acylations with the next amino acid in the sequence. Thus effective acylation times for each of the first six alanines are less than 60 min, while complete acylation with Ala_7, Ala_8, Ala_9, Ala_{10} is 26, 28, 30 and 7 hours, respectively (Fig. 2).

Kent (Ref. 2) proposes a number of solutions to the problem related to sequence-dependent coupling difficulties, viz. the use of heat in the coupling step and a quantitative conversion of residual unreacted resin-bound peptide chains to terminated species in a "capping" procedure.

Summary of the invention

The object of the present invention is to provide an improved SPPS according to which peptides, which are recognized as or prove to be "difficult sequences", can be synthesized in high yield and purity.

A further object of the invention is to provide an improved SPPS which provides for reduced coupling times, not only for difficult sequences, but also for otherwise uncomplicated sequences where it is desirable to reduce the normally long coupling times.

A still further object of the invention is to provide an agent or a kit for use in SPPS, whereby the above-mentioned process improvements are obtained.

The manner in which these and other objects and advantages of the invention is achieved will appear more fully from the following description and accompanying drawings showing hplc diagrams for various peptides obtained by solid phase synthesis.

Brief description of the drawings

Fig. 1 is an hplc of crude H-Ala$_{10}$-Lys-OH showing a substantial amount of deletion peptides (peak 1, 2, 3 and 4) and incompletely Fmoc-deprotected peptides (peak 6, 7, 8 and 9) besides the target peptide (peak 5).

Fig. 2 is a diagram showing sequential coupling times for each of the alanines in the synthesis of H-Ala$_{10}$-Lys-OH.

Fig. 3 is an hplc of H-Ala$_{10}$-Lys$_3$-OH showing the target peptide. No deletion peptides are observed.

Fig. 4 is an hplc of H-Ala$_{20}$-Lys$_3$-OH showing deletion peptides (peak 1, 2, 3, 4 and 5) besides the target peptide (peak 6).

Fig. 5 is an hplc of H-Ala$_{10}$-Lys$_6$-OH. No deletion peptides are observed.

Fig. 6 is an hplc of H-Ala$_{20}$-Lys$_6$-OH. No deletion peptides are observed.

Fig. 7 is an hplc of H-VQAAIDYING-OH, Acyl Carrier Protein (ACP) 65-74). The target peptide (peak

3) is accompanied with deletion peptides (peak 2 is the des-Val peptide).

Fig. 8 is an hplc of H-VQAAIDYING-K_6-OH, Acyl Carrier Protein (ACP) 65-74 coupled to the Lys(Boc)$_6$-pre-sequence with only a minor amount of des-Val peptide (peak 1).

Fig. 9 is an hplc of H-Ala_{10}-Lys-OH prepared by introduction of an HMPA linker. Several deletion peptides are observed (from Ala_{10}-Lys(tBOC)-HMPA-(Lys(tBoc))$_6$-resin).

Fig.10 is a similar hplc of H-Ala_{10}-Lys-OH prepared by using an MMa-linker showing a significant reduction the amount of deletion peptides compared to Fig. 9 (from Ala_{10}-Lys-MMa-(Lys(tBoc))$_6$-resin).

Detailed disclosure of the invention

To investigate the problems described above with reference to Ref. 3 and 4 further the present inventors addressed themselves to the question to which degree the β-sheet formation of the homo oligo-alanine chain may be affected by inclusion of a shorter peptide sequence, a pre-sequence, in the chain at the C-terminus. This question is associated with the fact that protein structures and polypeptide sequences may have stretches of well defined structures dependent on the amino acids in the sequence and of preceding amino acids. As mentioned earlier Chou and Fasmans investigations (Ref. 6) on protein structures have led to recognition of classes of amino acids which are defined as predominantly α-helix inducing, β-sheet or random coil inducing. Although it *a priori* may be assumed that similar predictions may apply for

homo oligo alanines or leucines it was pointed out, however, in Ref. 4 that Chou and Fasman rules do not predict homo oligo alanine or leucine peptides as β-sheet forming chains. Furthermore, Chou and Fasman's rules cannot be expected to apply during peptide synthesis on a resin and do clearly not apply when side-chain protected amino acids are part of the synthesis.

In the basic studies the synthesis of H-(Ala)n-(Lys)m-OH, where (Lys)m represent the pre-sequence with m equal to 1, 3 and 6 was investigated. A continuous-flow version of the polyamide solid-phase method (Ref. 7) on a fully automated peptide synthesizer developed was used as previously described (Ref. 14) with DMF as solvent and 3 times excess of Fmoc-alanine and Fmoc-lysine(tBoc)-pfp esters, respectively and standard Fmoc-deprotection with 20% piperidine in DMF for 10 minutes. Coupling times were monitored with Dhbt-OH which is deprotonated to the yellow Dbht-O$^-$ anion when un-acylated amino groups are still present and disappearance of the yellow colour marks the end-point of the synthesis. After cleavage of the peptide from the resin with 95% aqueous TFA, the product was washed with ether and analyzed by hplc. The results with m = 1 are described above (Fig. 1). In case of m = 3, it is seen from the hplc trace shown in Fig. 3 that the synthesis may be continued to Ala_{10} without detectable amounts of deletion peptides or incomplete Fmoc-deprotection. However, when continuing the synthesis to Ala_{20} the chromatogram (Fig. 4) shows the presence of deletion peptides. The results are even more striking with H-(Ala)n-$(Lys)_6$-OH where products without detectable deletion peptides are obtained with both Ala_{10} (Fig. 5) and Ala_{20} (Fig. 6). Furthermore, coupling times

are drastically reduced from up to 30 hours to standard coupling times (< 2 hours) in the single steps. The H-Ala$_{20}$-OH sequence has previously been attempted synthesized by the Boc-methodology but high levels of deletion and insertion peptides were obtained (Ref. 8). Clearly the pre-sequence Lys$_6$, which under the prevailing synthesis conditions is fully protected with the tBoc-group, has a most definitive and favourable effect on the structure of the growing peptide chain eliminating the otherwise very severe synthetic problems due to incomplete deprotections and extremely slow couplings.

In accordance with these surprising findings the present invention is based on the incorporation of a particular pre-sequence in the C-terminal part attached to the solid support.

This is a fundamental breach with the prior art attempts to deal with difficult sequences, where the focus was on the reaction conditions and the nature of the solid support.

As further discussed below the C-terminal sequence by which the desired peptide is attached to the solid support might also include suitable linkers in order to provide for e.g. better attachment or cleavage conditions.

Thus in a first aspect the present invention relates to a process for the production of peptides

$X\text{-}AA_1\text{-}AA_2 \ldots\ldots AA_n\text{-}Y$

wherein AA is an L- or D-amino acid residue, X is hydrogen or an amino protective group, Y is OH, NH_2 or an amino acid sequence comprising from 3 to 9 amino

acid residues and n is an integer greater than 2 by solid phase synthesis wherein the C-terminal amino acid in the form of an N-α-protected, if necessary side chain protected reactive derivative is coupled to a solid support or a polymer optionally by means of a linker, subsequently N-α-deprotected, whereafter the subsequent amino acids forming the peptide sequence are stepwise coupled or coupled as a peptide fragment in the form of suitably protected reactive derivatives or fragments, wherein the N-α-protective group is removed following formation of the desired peptide and the peptide is cleaved from the solid support, c h a r a c t e r i z e d in that the C-terminal part attached to the support or polymer comprises a pre-sequence comprising from 3 to 9, preferably from 5 to 7 amino acid residues independently selected from native L-amino acids having a side chain functionality which is protected during the coupling steps and having a propensity factor Pα > 0,57 and a propensity factor Pβ > 1,10 or the corresponding D-amino acids and said pre-sequence is optionally cleaved from the formed peptide.

L-amino acids meeting the above-mentioned limits for the propensity factors Pα and Pβ are Lys, Glu, Asp, Ser, His, Asn, Arg, Met and Gln.

These amino acids all have a side chain functionality selected from a carboxy, carboxamido, amino, hydroxy, guanidino, sulphide or imidazole group.

Presently preferred amino acids in the pre-sequence are Lys and Glu and combinations thereof, e.g. (Glu)q(Lys)p, where p + q is 3 to 9, preferably 6 to 9, and the order of Lys and Glu is arbitrarily chosen.

The N-α amino group of the amino acids or peptide fragments used in each coupling step should be suitably protected during the coupling. The protective group may be Fmoc or Boc or any other suitable protective group, e.g. those described above with reference to Ref. 13 and 18. The presently preferred N-α protective group is Fmoc.

It is important that the side chain functionality in the pre-sequence is suitably protected during the coupling steps. Such protective groups are well-known to a person skilled in the art and the preferred groups are listed in claims 7-12.

Without wishing to be bound by any particular theory, it is assumed that the physical-chemical properties of the protected side-chain of the pre-sequence exemplified by lysine are responsible for the observed "structural assisted peptide synthesis" (SAPS) by reducing or eliminating β-sheet formation in the poly alanine sequence.

In case of other homo-oligo pre-sequences it has been observed that (Glu(tBu))6, as well as the mixed sequence (Glu(tBu)Lys(tBoc))3 induces a favourable structure in the poly-alanine chain affording products without deletion peptides.

To investigate whether SAPS is a more general phenomenon or if it is confined only to homo-oligopeptides such as the poly alanine sequence, some mixed sequences reputedly known as difficult sequences were investigated.

The synthesis of H-VQAAIDYING-OH, Acyl Carrier Protein (ACP) 65 - 74, is a well known difficult synthesis which has been used as a model reaction in a num-

ber of cases (Ref. 9). Deletion peptides are observed in standard synthesis with a des-Val peptide (peak 2) as a prominent by-product (see example 7 and figure 7).

In accordance with the invention the sequence H-VQAAIDYING-K_6-OH was synthesized using the pre-sequence $(Lys(tBoc))_6$ attached at the C-terminus on a pepsyn K. The synthesis proceeded to give a product with the correct molecular weight in high purity and a significantly reduced amount of des-Val peptide (see example 8 and figure 8).

Another difficult sequence is reported to be H-VNVNVQVQVD-OH which has been synthesized on a variety of flow resins (Rapp polymer, PEG-PS, Pepsyn K, PEGA 1900/300, PEGA 800/130 and PEGA 300/130) in all cases accompanied by considerable glutamine preview already from Val^1 except when using a PEGA 1900/300 resin (Ref. 10). In accordance with the invention the synthesis of H-VNVNVQVQVDK_6-OH proceeded to give a product with the correct molecular weight. Deletion peptides were not detected in the spectrum (see example 9).

In order to verify another important aspect of the present invention, viz. the reduced coupling times obtained by introducing a pre-sequence at the C-terminal part of desired peptide the coupling times of the individual amino acids in the synthesis of enkephalin, H-Tyr-Gly-Gly-Phe-Leu-OH, have been monitored with and without the pre-sequence (Lys(tBoc))6 as described in example 15.

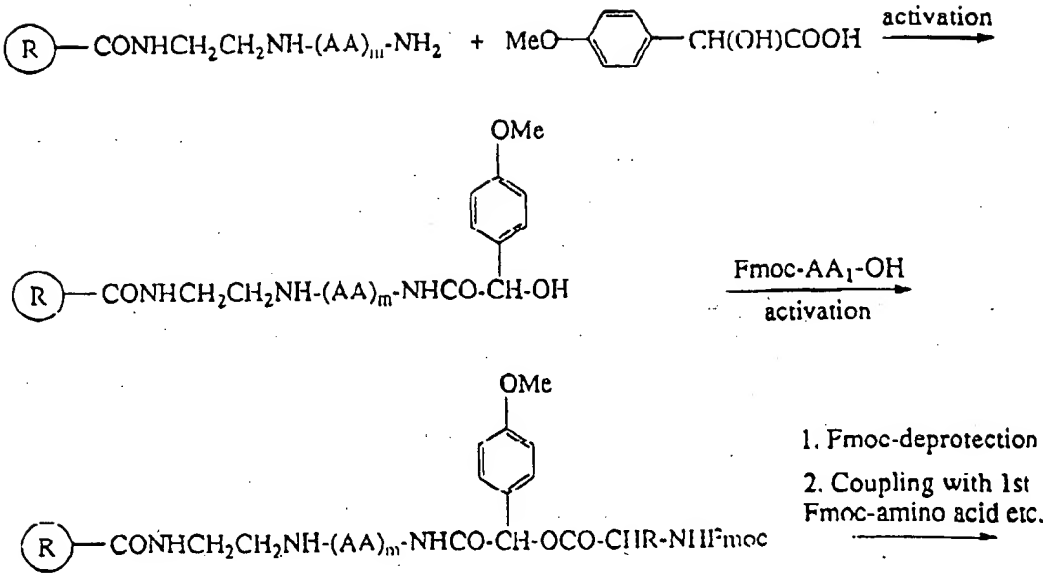
The results of the measurements demonstrate that the coupling times in this otherwise uncomplicated syn-

thesis proceeds effectively in combination with the chosen pre-sequence.

In the above described cases the peptide sequences have been obtained with a hexa lysine pre-sequence which for certain purposes may be acceptable or even of advantage. Thus, a β-sheet forming sequence may cause severe solubility problems, but in case of H-$Ala_{20}(Lys)_6$-OH the peptide turned out to be soluble in aqueous solutions while H-Ala_{10}-Lys-OH very rapidly precipitated from TFA solutions when diluted with water. In case of $(Glu)_6$ the solubility provided by the pre-sequence and the multitude of carboxylic acid groups may be utilized for e.g. ELISA, where site-directed attachment to a suitably amino-group-activated surface is possible because of facile activation in aqueous solution with e.g. a carbodiimide. However, it has also been considered, how the observations may be utilized for practical peptide synthesis without presence of the pre-sequence in the final product. It was therefore attempted to introduce a linker between the pre-sequence and the target peptide, e.g. resin-$(Lys(tBoc))_6$-linker-peptide, permitting cleavage of the peptide from the linker with standard reagents such as scavenger containing TFA solutions. Introduction of the commonly used HMPA linker as described below in example 10, resin-$(Lys(tBoc))_6$-HMPA-$Lys(tBoc)Ala_{10}$, has, however, a clear negative effect on the synthesis of H-Ala_{10}-Lys-OH giving rise to formation of deletion peptides (Fig. 9). Apparently, the effect of the pre-sequence on the poly alanine structure is lost, pointing to that the aromatic group in the linker breaks the structure effect in the peptide back-bone of $(Ala)_n$-$(Lys)_m$. In order to conserve the structural effect the introduction of an optically active linker be-

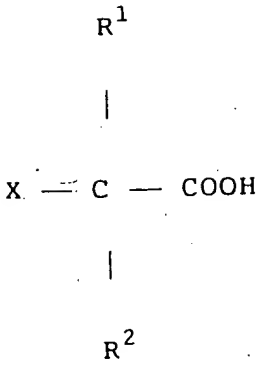
tween $(Ala)_n$ and $(Lys)_m$ was investigated using 4-methoxymandelic acid (MMa) as a possible candidate. This type of linker has supposedly not been used hitherto. The presence of the methoxy group is assumed to facilitate the cleavage of the target peptide from the linker by means of standard scavenger containing TFA solutions. 4-Methoxymandelic acid may be resolved in its optically active forms of which the R-configuration ((+)-configuration) is identical to L-protein amino acids. Two types of experiments have been carried out: (a) synthesis using racemic 4-methoxymandelic acid; (b) synthesis using R- 4-methoxymandelic acid (R-MMa). The synthesis scheme is shown below (Scheme 1) and further illustrated in examples 11, 12, 13 and 14.

Scheme 1.


R—CONHCH2CH2NH-(AA)m-NH2 + MeO— —CH(OH)COOH
activation
OMe
R—CONHCH2CH2NH-(AA)m-NHCO-CH-OH
Fmoc-AA1-OH
activation
OMe
R—CONHCH2CH2NH-(AA)m-NHCO-CH-OCO-CHR-NHFmoc
1. Fmoc-deprotection
2. Coupling with 1st Fmoc-amino acid etc.

In the first case the sequence resin-(Lys(tBoc))$_6$-MMa-Lys-Ala$_{10}$ was synthesized to give H-Lys-Ala$_{10}$-OH where the lysine group is introduced to enhance solubility in aqueous solutions for hplc analysis. The results of the hplc analysis is shown in Fig. 10. A much better product was achieved than for the HMPA-linker (Fig. 9 and example 10) although deletion peptides are present. In case (b) the same peptide was synthesized under identical conditions and a peptide is formed without detectable deletion peptides or incomplete Fmoc-deprotection. These results may be compared to synthesis of H-Lys-Ala$_{10}$-OH using the construct resin-MMa-Lys-Ala$_{10}$ where the pre-sequence Lys(tBoc))$_6$ is omitted. Significant amounts of deletion peptides are noted.

In accordance with the above observations another aspect of the present invention relates to a method for the solid phase synthesis of peptides as described above having the further feature that a linker is inserted between the pre-sequence attached to the support and the desired peptide sequence AA_1-AA_n, which enables a selective cleavage of said sequence. Preferably, the linker is optically active. An applicable group of linkers is α-hydroxy and α-amino acids of the general formula



wherein X is OH or NH_2, and R^1 and R^2 are independently selected from H, C_{1-3} alkyl, phenyl and substituted phenyl, where the substitutents are one or more electron donating substituents chosen among C_{1-3} alkoxy, C_{1-3} alkyl, or two vicinal substituent groups are joined to form a 5 or 6 membered ring together with the carbon atoms to which they are attached.

The most preferred linkers are racemic 4-methoxymandelic acid, (+)-4-methoxymandelic acid, diphenylglycin and glycolic acid.

It will be understood that when X is OH the product formed after cleavage will be a peptide AA_1-AA_n-OH, i.e. Y = OH, while in case of X being NH_2 a peptide amide AA_1-AA_n-NH_2 is formed, i.e. Y = NH_2

In a still further embodiment of the invention a first linker is inserted between the pre-sequence attached to the support and the AA_1-AA_n sequence and a second linker is inserted between the pre-sequence and the solid support with orthogonal cleavage conditions to the first linker enabling a selective cleavage of the second linker, e.g. by means of trifluoroacetic acid (TFA), trifluoromethanesulfonic acid (TFMSA), HBr, HCl, HF or a base such as ammonia, hydrazine, alkoxide or hydroxide to give the desired peptide AA_1-AA_n linked to the pre-sequence by means

of said first linker, which is then optionally cleaved from the pre-sequence

Another important embodiment of the invention relates to agents for use in solid phase synthesis incorporating one or more of the above described features with relation to pre-sequences and linkers.

A first aspect of this embodiment relates to an agent for use in solid phase peptide synthesis having the general formula

$X\text{-}AA'_1\text{-}...\text{-}AA'_m\text{-}Y_1\text{-}R$

wherein R is a solid support applicable in solid phase peptide synthesis, Y_1 is an amino acid sequence comprising from 3 to 9, preferably from 5 to 7 amino acid residues independently selected from native L-amino acids having a side chain functionality which is protected during the coupling steps and having a propensity factor $P\alpha > 0,57$ and a propensity factor $P\beta \leq 1,10$, e.g. Lys, Glu, Asp, Ser, His, Asn, Arg, Met or Gln, or the corresponding D-amino acid, AA' is an L- or D-amino acid residue, m is zero or an integer from 1 to 40 and X is hydrogen or an amino protective group.

A second aspect of this embodiment relates to an agent for use in solid phase peptide synthesis having the general formula

$X\text{-}AA'_1\text{-}...\text{-}AA'_m\text{-}L_1\text{-}Y_1\text{-}R$

wherein X, AA', m, Y_1 and R are as defined above and L_1 is a preferably optically active linker which enables a selective cleavage of the bond to AA'_m.

Preferred linkers are α-hydroxy or α-amino acids as described above.

A third aspect of this embodiment relates to an agent for use in solid phase peptide synthesis having the general formula

$X\text{-}AA'_1\text{-}\ldots\text{-}AA'_m\text{-}L_1\text{-}Y_1\text{-}L_2\text{-}R$

wherein X, AA', m, Y_1, R and L_1 are as defined above and L_2 is a linker which enables a selective cleavage from the solid support.

A fourth aspect of this embodiment relates to an agent for use in solid phase peptide synthesis having the general formula

$X\text{-}AA'_1\text{-}\ldots\text{-}AA'_m\text{-}Y_1\text{-}L_2\text{-}R$

wherein X, AA', m, Y_1, R and L_2 is as defined above.

It will be understood that the above described agents may be in the form of polymers, gels or other solid phases which are prepared for solid phase peptide synthesis according to the invention by

a) containing a pre-sequence and optionally one or more amino acids from the desired sequence (the first aspect) or

b) containing a pre-sequence, a preferably optically active cleavage linker and optionally one or more amino acids from the desired sequence (the second aspect), or

c) containing a second linker enabling cleavage from the support, a pre-sequence, a first preferably optically active cleavage linker and optionally one

or more amino acids from the desired sequence (the third aspect) and

d) containing a linker enabling cleavage from the support, a pre-sequence and optionally one or more amino acids from the desired sequence (the fourth aspect).

The specific conditions used in the experiments on which this invention is based are stated below under general procedures.

Generally speaking, apart from the novel and characteristic features related to the pre-sequence and the novel cleavage linkers the method according to the invention may be carried out under traditional conditions for solid phase peptide synthesis described in the literature referred to in the background art.

However, a brief summary is deemed proper.

Fmoc groups may be deprotected by means of an amine such as piperidine or diazabicyclo[5,4,0]undec-7-ene (DBU).

Side chain protective groups may be deprotected by means of an acid such as trifluoroacetic acid (TFA), trifluoromethanesulfonic acid (TFMSA), HBr, HCl or HF.

The solid support is preferably selected from functionalized resins such as polystyrene, polyacrylamide, polyethyleneglycol, cellulose, polyethylene, latex or dynabeads.

If desired, C-terminal amino acids may be attached to the solid support by means of a common linker such as 2,4-dimethoxy-4'-hydroxy-benzophenone, 4-(4-hydroxy-

methyl-3-methoxyphenoxy)-butyric acid (HMPB), 4-hydroxymethylbenzoic acid, 4-hydroxymethylphenoxy-acetic acid (HMPA), 3-(4-hydroxymethylphenoxy)propionic acid or p-[(R,S)-a[1-(9H-fluoren-9-yl)-methoxyformamido]-2,4-dimethoxybenzyl]-phenoxyacetic acid (AM).

The synthesis may be carried out batchwise or continuously on an automated or semi automated peptide synthesizer.

The individual coupling steps may be performed in the presence of a solvent, e.g. selected from acetonitrile, N,N-dimethylformamide (DMF), N-methylpyrrolidone (NMP), dichloromethane (DCM), trifluoroethanol (TFE), ethanol, methanol, water, mixtures of the mentioned solvents with or without additives such as perchlorate or ethylenecarbonate.

The individual couplings between two amino acids, an amino acid and the earlier formed peptide sequence or a peptide fragment and the earlier formed peptide sequence may be carried out according to usual condensation methods such as the azide method, mixed acid anhydride method, symmetrical anhydride method, carbodiimide method, active ester method such as pentafluorophenyl(Pfp), 3,4-dihydro-4-oxobenzotriazin-3-yl (Dhbt), benzotriazol-1-yl (Bt), 7-azabenzotriazol-1-yl (At), 4-nitrophenyl, N-hydroxysuccinic acid imido esters (NHS), acid chlorides, acid fluorides, in situ activation by O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU), O-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), O-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate

(TBTU), or benzotriazolyl-oxy-tris-(dimethylamio)-phosphonium hexafluorophosphate (BOP).

The formed peptide may be cleaved from the support by means of an acid such as trifluoroacetic acid (TFA), trifluoromethanesulfonic acid (TFMSA), hydrogen bromide (HBr), hydrogen chloride (HCl), hydrogen fluoride (HF) or a base such as ammonia, hydrazine, an alkoxide or a hydroxide.

Alternatively, the peptide is cleaved from the support by means of photolysis.

In the embodiment, where a linker is inserted between the pre-sequence attached to the support and the AA_1-AA_n sequence which enables a selective cleavage of said sequence, said cleavage may be made by means of an acid such as trifluoroacetic acid (TFA), trifluoromethanesulfonic acid (TFMSA), hydrogen bromide (HBr), hydrogen chloride (HCl), hydrogen fluoride (HF) or a base such as ammonia, hydrazine, an alkoxide or a hydroxide.

Sequence Assisted Peptide Synthesis (SAPS).

EXPERIMENTAL PROCEDURES:

Peptide synthesis

General procedures

Apparatus and synthetic strategy

Peptides were synthesized either batchwise in a polyethylene vessel equipped with a polypropylene filter for filtration or in the continuous-flow version of the polyamide solid-phase method (Dryland, A. and Sheppard, R.C., Ref. 7) on a fully automated peptide

synthesizer (Cameron et al., Ref. 14) using 9-fluorenylmethyloxycarbonyl (Fmoc) or tert.-Butyloxycarbonyl, (Boc) as N-α-amino protecting group and suitable common protection groups for side-chain functionalities.

Solvents

Solvent DMF (*N*,*N*-dimethylformamide, Riedel de-Häen, Germany) was purified by passing through a column packed with a strong cation exchange resin (Lewatit S 100 MB/H strong acid, Bayer AG Leverkusen, Germany) and analyzed for free amines prior to use by addition of 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (Dhbt-OH) giving rise to a yellow color (Dhbt-O^- anion) if free amines are present. Solvent DCM (dichloromethane, analytical grade, Riedel de-Häen, Germany) was used directly without purification.

Amino acids

Fmoc-protected amino acids and corresponding pentafluorophenyl (Pfp) esters were purchased from MilliGen, UK, NovaBiochem, Switzerland and Bachem, Switzerland, and the Dhbt-esters from NovaBiochem, Switzerland in suitable side-chain protected forms. Boc-protected amino acids were purchased from Bachem, Switzerland.

Coupling reagents

Coupling reagent diisopropylcarbodiimide (DIC) was purchased from Riedel de-Häen, Germany and distilled prior to use, dicyclohexylcarbodiimide (DCC) was purchased from Merck-Schuchardt, München, Germany, and purified by destillation. O-Benzotriazolyl-N,N,N',N'-tetramethyluronium tetrafluoroborate (TBTU) was pur-

chased from PerSeptive Biosystems GmbH Hamburg, Germany.

Linkers

Linkers HMPA, Novabiochem, Switzerland; 4-hydroxymethylbenzoic acid, Novabiochem; 4-methoxymandelic acid, Aldrich, Germany; HMPB, Novabiochem; AM, Novabiochem; 3-(4-hydroxymethylphenoxy)propionic acid, Novabiochem, was coupled to the resin as a preformed 1-hydroxybenzotriazole (HObt) ester generated by means of DIC. Racemic 4-methoxymandelic acid (98% pure, Aldrich, Germany) was used directly as linker or resolved by treatment with (+)-cinchonine (85% pure, Aldrich, Germany) giving the optical active linker (+)-4-methoxymandelic acid, $[a]^{20}$ =+ 146 (water) in 95.8 % optical purity and (-)-4-methoxymandelic acid, $[a]^{20}$ =- 128.6 (water) in 88.1 % optical purity.

Resolution of (+/-)-4-methoxymandelic acid (A. Mc Kenzie, D.J.C. Pirie, Ref. 16; E. Knorr, Ref. 17)

(+/-)-4-Methoxymandelic acid (10 g, 54.89 mmol; Aldrich, 98%) was dissolved in 500 ml hot water (60-80 ^{o}C) and the solution decanted while still warm in order to remove insoluble impurities. (+)-Cinchonine (16,16 g, 54.89 mmol, Aldrich, 85%, $[a]_D^{20}$ = + 211 ^{o} (litt.: + 228 ^{o})) was added to the hot solution in small portions. The solution became clear after 15 min stirring at 60-80 ^{o}C and was cooled in ice. After 1 h the precipitate was collected by filtration and dried in an exsiccator over night, yielding 9.9 g of the chinconine salt. The salt was recrystallized from boiling water (80 ml), the solution decanted while still warm and then cooled in ice. The precipitate was collected by filtration after 1 h, washed three

times with cold water, and dried in an exsiccator over night yielding 7.84 g (16.45 mmol). The chinconine salt (2 g, 4,2 mmol) was dissolved in 40 ml 2N HCl and immediately extracted with 3 x 30 ml diethylether. The ether phase was dried over Na_2SO_4 and evaporated to dryness yielding 0.55 g 4-methoxymandelic acid. The optical purity of the liberated 4-methoxymandelic acid was estimated to 18.5% ($[a]_D^{20}$ = + 27 °). After a second recrystallisation of the chinconine salt followed by liberation of the mandelic acid as described above the optical purity was estimated to 69.0 % ($[a]_D^{20}$ = + 100.8 °). A third recrystallisation resulted in an optical purity of 95,8% ($[a]_D^{20}$ = + 140.0 °).

Solid supports

Peptides synthesized according to the Fmoc-strategy were synthesized on three different types of solid support using 0.05 M or higher concentrations of Fmoc-protected activated amino acid in DMF. 1) PEG-PS (polyethyleneglycol grafted on polystyrene; TentaGel S NH_2 resin, 0.27 mmol/g, Rapp Polymere, Germany or NovaSyn TG resin, 0.29 mmol/g, Novabiochem, Switzerland) ; 2) PepSyn Gel (polydimethylacrylamide resin functionalized with sarcosine methylester, 1.0 mmol/g; MilliGen, UK). 3) PepSyn K (Kieselguhr supported polydimethylacrylamide resin functionalized with sarcosine methylester 0.11 mmol/g; MilliGen, UK).

Peptides synthesized according to the Boc-strategy were synthesized on a Merrifield-resin (polystyrene-divinylbenzene) with the first amino acid attached (Novabiochem, Switzerland).

Catalysts and other reagents

Diisopropylethylamine (DIEA) was purchased from Aldrich, Germany, and ethylenediamine from Fluka, Switzerland, piperidine from Riedel-de Häen, Frankfurt, Germany. 4-(N,N-dimethylamino)pyridine (DMAP) was purchased from Fluka, Switzerland and used as a catalyst in coupling reactions involving symmetrical anhydrides. 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (Dhbt-OH) was obtained from Fluka, Switzerland, and 1-hydroxybenzotriazole (HObt) from NovaBiochem, Switzerland.

Coupling procedures

The first amino acid was coupled as a symmetrical anhydride in DMF generated from the appropriate N-α-protected amino acid and DIC. The following amino acids were coupled as Pfp- or Dhbt-esters or as preformed HObt esters made from appropriate N-α-protected amino acids and HObt by means of DIC or TBTU in DMF. In the case of Fmoc all acylations were checked by the ninhydrin test performed at 80 °C in order to prevent Fmoc deprotection during the test (Larsen, B. D. and Holm, A., Ref. 4)

Deprotection of the N-α-amino protecting group.

Deprotection of the Fmoc group was performed by treatment with 20% piperidine in DMF (1x3 and 1x7 min when synthesized batchwise) or by flowing the deprotection solvent through the resin (10 min, flow rate 1 ml/min using continuous flow synthesis), followed by wash with DMF until no yellow color ($Dhbt\text{-}O^-$) could be detected after addition of Dhbt-OH to the drained DMF.

Deprotection of the Boc group was performed by treatment with 50% TFA in DCM (v/v) 1x1.5 min and 1x20 min followed by wash 6x9 min each with DCM, neutralization with 10% triethylamine in DCM (v/v) 2x1.5 min each, followed by 6x9 min wash with DCM.

Cleavage of peptide from resin with acid.

Peptides were cleaved from the resins by treatment with 95% triflouroacetic acid (TFA, Halocarbon Products Corporation, U.S.A.; Biesterfeld & Co. Hamburg, Germany)-water v/v at r.t. for 2 h. The filtered resins were washed with 95% TFA-water and filtrates and washings evaporated under reduced pressure. The residue was washed with ether and freeze dried from acetic acid-water. The crude freeze dried product was analyzed by high-performance liquid chromatography (hplc) and identified by matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI TOF MS) or by electrospray ionization mass spectrometry (ESMS).

Cleavage of peptide from resin with base.

The dried resin (1 g) was treated with 1M sodium hydroxide (10 ml) at 4 oC and left for 15 min at rt. The resin was filtered into a flask containing 10% aq. acetic acid. The peptide was isolated by lyophilization and submitted to gel filtration.

Cleavage of peptide from resin with TFMSA.

The dried resin (250 mg) was placed in a round-bottomed flask with a stirring bar. Thioanisole/ethanedithiol (2:1, 750 µl) was added, the mixture chilled in ice, 5 ml TFA was added and the mixture was stirred for 5 - 10 min. TFMSA (500 µl) was

added dropwise and the reaction continued at rt for 30 - 60 min. The peptide was precipitated after addition of ether.

Deprotection of side chain protective groups

Preferably, the side chains are deprotected simultaneously with the cleavage of the peptide from the resin.

Preformed HObt-ester

Method a. 3 eq. N-α-amino protected amino acid was dissolved in DMF together with 3 eq. HObt and 3 eq DIC. The solution was left at r.t. for 10 minutes and then added to the resin, which had been washed with a solution of 0.2% Dhbt-OH in DMF prior to the addition of the preactivated amino acid.

Method b. 3 eq. N-α-amino protected amino acid was dissolved in DMF together with 3 eq. HObt, 3 eq TBTU and 4,5 eq. DIEA. The solution was left at r.t. for 5 minutes and then added to the resin.

Preformed symmetrical anhydride

6 eq. N-α-amino protected amino acid was dissolved in DCM and cooled to 0 °C. DCC (3 eq.) was added and the reaction continued for 10 min. The solvent was removed *in vacuo* and the remanens dissolved in DMF. The solution was filtered and immediately added to the resin followed by 0.1 eq. of DMAP.

Estimation of the coupling yield of the first N-α-amino protected amino acid

3 - 5 mg dry Fmoc-protected peptide-resin was treated with 5 ml 20% piperidine in DMF for 10 min at r.t.

and the UV absorption for the dibenzofulvene-piperidine adduct was estimated at 301 nm. The yield was determined using a calculated extension coefficient e301 based on a Fmoc-Ala-OH standard.

In case of Boc-protection, the coupling was estimated according to the ninhydrin-method after removal of the Boc-group (Sarin, V.K. et al., Ref. 11)

Peptide synthesis on PepSyn K resin

Dry PepSyn K (ca 500 mg), was covered by ethylenediamine and left at rt over night. The resin was drained and washed with DMF 10 x 15 ml, 5 min each. After draining the resin was washed with 10% DIEA in DMF v/v (2 x 15 ml, 5 min each) and finally washed with DMF until no yellow color could be detected by addition of Dhbt-OH to the drained DMF. 3 eq. HMPA 3 eq. HObt and 3 eq. DIC was dissolved in 10 ml DMF and left for activation for 10 min, after which the mixture was added to the resin and the coupling continued for 24 h. The resin was drained and washed with DMF (10 x 15 ml, 5 min each), and the acylation was checked by the ninhydrin test. The first amino acid was coupled as the side chain protected preformed symmetrical anhydride (see above), and the coupling yields estimated as described above. It was in all cases better than 70%. The synthesis was then continued either as "*continuous-flow* " or as "*batchwise* " as described below.

Continued peptide synthesis on PepSyn K using continuous-flow technique.

The resin (ca. 500 mg) with the first amino acid attached was placed in a column connected to the fully automated peptide synthesizer. The Fmoc group was

deprotected as described above. The remaining amino acids according to the sequence were coupled as Fmoc-protected, if necessary side chain protected, Pfp esters (3 eq.) with the addition of Dhbt-OH (1 eq.). The end-point of each coupling was determined automatically by monitoring the disappearance of the yellow color of the Dhbt-OH anion spectrophotometrically. After completed synthesis the peptide-resin was washed with DMF (10 min flow rate 1 ml/min), DCM (3x5 ml, 3 min each) and finally diethyl ether (3x5 ml each), removed from the column and dried *in vacuo*.

Continued batchwise peptide synthesis on PepSyn K.

The resin (ca. 500 mg) with the first amino acid attached was placed in a polyethylene vessel equipped with a polypropylene filter for filtration, and the Fmoc-group deprotected as described above. The remaining amino acids according to the sequence were coupled as preformed Fmoc-protected, if necessary side chain protected, HObt esters (3 eq.) in DMF (5 ml) prepared as described above. The couplings were continued for 2 h unless otherwise specified. Excess reagent was then removed by DMF washing (12 min, flow rate 1 ml/min) All acylations were checked by the ninhydrin test performed at 80 ^{o}C. After completed synthesis the peptide-resin was washed with DMF (1o min, flow rate 1 ml/min), DCM (5x5 ml, 1 min each) and finally diethyl ether (5x5 ml, 1 min each) and dried *in vacuo*.

Batchwise peptide synthesis on PEG-PS.

TentaGel S NH_2 or NovaSyn TG resin (250 mg, 0.27-0.29 mmol/g) was placed in a polyethylene vessel equipped with a polypropylene filter for filtration. The resin was swelled in DMF (5 ml), and treated with 20%

piperidine in DMF to secure the presence of non-protonated amino groups on the resin. The resin was drained and washed with DMF until no yellow color could be detected after addition of Dhbt-OH to the drained DMF. HMPA (3 eq.) was coupled as a preformed HObt-ester as described above and the coupling was continued for 24 h. The resin was drained and washed with DMF (5 x 5 ml, 5 min each) and the acylation checked by the ninhydrin test. The first amino acid was coupled as a preformed symmetrical anhydride as described above. The coupling yields of the first Fmoc-protected amino acids were estimated as described above. It was in all cases better than 60%. The following amino acids according to the sequence were coupled as preformed Fmoc-protected, if necessary side chain protected, HObt esters (3 eq.) as described above. The couplings were continued for 2 h, unless otherwise specified. The resin was drained and washed with DMF (5 x 5 ml, 5 min each) in order to remove excess reagent. All acylations were checked by the ninhydrin test performed at 80 °C. After completed synthesis the peptide-resin was washed with DMF (3x5 ml, 5 min each), DCM (3x5 ml, 1 min each) and finally diethyl ether (3x5 ml, 1 min each) and dried *in vacuo*.

Batchwise peptide synthesis on PepSyn Gel.

Dry PepSyn Gel resin (500 mg, 1 mmol/g) was placed in a polyethylene vessel equipped with a polypropylene filter for filtration. The resin was swelled in ethylenediamine (15 ml) and gently agitated by shaking for 20 h. The resin was drained and washed with DMF (10 x 15 ml, 5 min each). After draining the resin was washed with 10% DIEA in DMF v/v (2 x 15 ml, 5 min each) and finally washed with DMF (5 x 15 ml, 5

min each) until no yellow color could be detected after addition of Dhbt-OH to the drained DMF. HMPA (3 eq.) was coupled as a preactivated HObt-ester as described above (method a) and the coupling was continued for 24 h. The resin was drained and washed with DMF (5 x 15 ml, 5 min each). The acylation was checked by the ninhydrin test. The first amino acid was coupled as preformed side chain protected symmetrical anhydride as described above. The coupling yields of the first Fmoc-protected amino acids were estimated as described above. It was in all cases better than 70%. The remaining amino acids according to the sequence were coupled as preformed Fmoc-protected, if necessary side chain protected, HObt esters (3 eq.) as described above (method a). The couplings were continued for 2 h and, if necessary, double coupled over night. The resin was drained and washed with DMF (5 x 5 ml, 5 min each) in order to remove excess reagent. All acylations were checked by the ninhydrin test performed at 80 °C. The Fmoc group was deprotected as described above. After completed synthesis the peptide-resin was washed with DMF (3x15 ml, 5 min each), DCM (3x15 ml, 2 min each) and finally diethyl ether (3x15 ml, 2 min each) and dried *in vacuo*.

Hplc conditions.

Hplc was performed on a Waters 600 E instrument equipped with a Waters 996 Photodiode array detector with a Waters Radial Pak 8 x 100 mm C_{18} reversed-phase column. Buffer A was 0.1 vol % TFA in water and buffer B 90 vol% acetonitrile, 9.9 vol% water and 0.1 vol% TFA. The Buffers were pumped through the column at a flow rate of 1.5 ml/min using the gradient: 1. Linear gradient from 0% - 70% B (20 min), linear

gradient from 70 - 100% B (1 min), isocratic 100% B (5 min). 2. Isocratic with 0% B (2 min), linear gradient from 0 - 50% B (23 min), linear gradient from 50 - 100% B (5 min), isocratic 100% B (5 min).

Mass spectroscopy.

Matrix assisted laser desorbtion ionization time-of-flight (MALDI TOF) mass spectra were obtained on a Fisons TofSpec E instrument. Electrospray ionization mass spectra were obtained on a Finnigan Mat LCQ instrument equipped with an electrospray (ESI) probe (ES-MS).

Peptide synthesis of individual peptides.

Example 1. Synthesis of H-Ala$_{10}$-Lys-OH (comparison).

500 mg Fmoc-Lys(Boc) PepSyn KA resin (0.086 mmol/g) was used for synthesis according to "continuous-flow technique".

The crude freeze dried product was analyzed by hplc and found to be a complicated mixture comprising the target peptide (n = 10) as well as deletion peptides corresponding to n = 6, 7, 8, and 9 and deletion peptides with the Fmoc group still attached to the N-terminal where n = 6,7,8, and 9 respectively. The identity of the individual peptides was confirmed by MALDI TOF MS.

Example 2. Synthesis of H-Ala$_{10}$-Lys$_3$-OH.

500 mg Fmoc-Lys(Boc) PepSyn KA resin (0.086 mmol/g) was used for synthesis according to "continuous-flow technique".

The crude freeze dried product was analyzed by hplc and found to be homogeneous without deletion and Fmoc-protected sequences. Yield 91,0%. The purity was found to be better than 98% according to hplc (see Fig. 3). The identity of the peptide was confirmed by MALDI TOF MS.

Example 3. Synthesis of H-Ala$_{10}$-Lys$_6$-OH.

500 mg Fmoc-Lys(Boc) PepSyn KA resin (0.086 mmol/g) was used for synthesis according to "continuous-flow technique".

The crude freeze dried product was analyzed by hplc and found to be homogeneous without deletion and Fmoc-protected sequences. Yield 90,9%. The purity was found to be better than 98% according to hplc (see Fig. 5). The identity of the peptide was confirmed by ES MS.

4. Synthesis of H-Ala$_{20}$-Lys$_3$-OH.

500 mg Fmoc-Ala$_{10}$-(Lys(Boc))$_3$ PepSyn KA resin (from the synthesis of H-Ala$_{10}$-Lys$_3$-OH) was used for synthesis according to "continuous-flow technique" and the synthesis continued.

The crude freeze dried product was analyzed by hplc and found to comprise the target peptide H-Ala$_n$-Lys$_3$-OH (n = 20) as well as deletion peptides corresponding to n = 19, 18, 17 and 16 (see Fig. 4). Fmoc-protected sequences were not detected. The identity of the peptides were confirmed by ES MS.

Example 5. Synthesis of H-Ala$_{20}$-Lys$_6$-OH.

500 mg Fmoc-Lys(Boc) PepSyn KA resin (0.086 mmol/g) was used for synthesis according to "continuous-flow technique".

The crude freeze dried product was analyzed by hplc and found to be homogeneous without deletion and Fmoc-protected sequences. Yield 91.4%. The purity was found to be better than 98% according to hplc (see Fig. 6). The identity of the peptide was confirmed by ES MS.

Example 6. Synthesis of H-Ala$_{20}$-Lys-(Glu-Lys)$_3$ -OH.

500 mg Fmoc-Lys(Boc) PepSyn KA resin (0.086 mmol/g) was used for synthesis according to "continuous-flow technique".

The crude freeze dried product was analyzed by hplc. It was found to be better than 98% pure without deletion and Fmoc-protected sequences. Yield 97%. The identity of the peptide was confirmed by ES MS.

Example 7. Synthesis of Acyl Carrier Protein (ACP) 65-74, H-Val-Gln-Ala-Ala-Ile-Asp-Tyr-Ile-Asn-Gly-OH (comparison).

500 mg Fmoc-Gly PepSyn KA resin (0.074 mmol/g) was used for synthesis according to "continuous-flow technique".

The crude freeze dried product was analyzed by hplc and found to contain the target molecule accompanied by ca. 16% of the des-Val peptide. The identity of the peptides was confirmed by MALDI TOF MS.

Example 8. Synthesis of Acyl Carrier Protein (ACP) 65-74, H-Val-Gln-Ala-Ala-Ile-Asp-Tyr-Ile-Asn-Gly-Lys_6-OH, using the pre-sequence $(Lys(Boc))_6$.

500 mg Fmoc-Lys(Boc) PepSyn KA resin (0.086 mmol/g) was used for synthesis according to "continuous-flow technique".

The crude freeze dried product was analyzed by hplc and found to contain the target peptide in high purity (~ 95%) with a significantly reduced amount of the des-Val peptide. The identity of the peptide was confirmed by MALDI TOF MS.

Example 9. Synthesis of H-Val-Asn-Val-Asn-Val-Gln-Val-Gln-Val-Asp-Lys_6-OH, using the pre-sequence $(Lys(Boc))_6$.

500 mg Fmoc-Lys(Boc) PepSyn KA resin (0.086 mmol/g) was used for synthesis according to "continuous-flow technique".

The crude freeze dried product was analyzed by hplc and found to be better than 90% pure without deletion and Fmoc-protected sequences. Yield 100%. The identity of the peptide was confirmed by MALDI TOF MS.

Example 10. Synthesis of H-Ala_{10}-Lys-OH using $(Lys(Boc))_6$ as pre-sequence and HMPA as linker (H-Ala_{10}-Lys(Boc)-OCH_2-$PhOCH_2CO$-$(Lys(Boc))_6$-$NHCH_2CH_2NH$ PepSyn K).

500 mg dry PepSyn K (0.1 mmol/g) was covered by ethylenediamine (5 ml) and left at rt over night. The resin was drained and washed with DMF 10 x 15 ml, 5 min each. After draining the resin was washed with 10% DIEA in DMF v/v (2 x 15 ml, 5 min each) and finally washed with DMF until no yellow color could be

detected by addition of Dhbt-OH to the drained DMF. The derivatized resin was used for synthesis according to "continuous-flow technique".

The first 6 Lysines forming the pre-sequence were coupled as Fmoc-Lys(Boc)-Pfp esters (3 eq.) with the addition of Dhbt-OH (1 eq.). The end-point of each coupling was determined automatically as described above. The Fmoc group was cleaved as described above. After finishing the pre-sequence 3 eq. HMPA coupled as a preactivated HObt ester as described above was introduced at the top of the column. The synthesizer was operated in recirculation mode for 2 h and excess of reagent was then removed by DMF washing (12 min, flow rate 1 ml/min). A small resin-sample was removed in order to check the acylation by the ninhydrin test. The next amino acid according to the sequence was coupled as preformed side chain protected symmetrical anhydride as described above and introduced at the top of the column together with (0.1 eq.) DMAP and the synthesizer was operated in recirculation mode for 90 min. Excess reagent was then removed by DMF washing (12 min, flow rate 1 ml/min). A small resin-sample was removed in order to check the coupling yield, which was estimated as earlier described and found to be 84%. The synthesis was then continued by cleavage of the Fmoc group as described above. The remaining amino acids according to the sequence were coupled as Fmoc-protected, if necessary side chain protected, Pfp esters (3 eq.) with addition of Dhbt-OH (1 eq.). The end-point of each coupling was determined automatically as described above. After completed synthesis the peptide-resin was washed with DMF (10 min, flow rate 1 ml/min), DCM (3x5 ml, 1 min each) and finally diethyl ether (3x5 ml, 1 min each), removed from the column and dried *in vacuo*.

The peptide was cleaved from the resin as described above.

The crude freeze dried product was analyzed by hplc and found to comprise the target peptide H-Ala$_n$-Lys-OH (n = 10) as well as deletion peptides corresponding to n = 9, 8, 7 and 6 (see Fig. 9). Fmoc-protected sequences were not detected. The identity of the peptides were confirmed by MALDI TOF MS.

Example 11. Synthesis of H-Ala$_{10}$-Lys-OH using (+/-)-4-methoxymandelic acid as linker (H-Ala$_{10}$-Lys(Boc)-OCH-(4-MeOPh)CONHCH$_2$CH$_2$NH PepSyn K resin) .

500 mg dry PepSyn K (0.1 mmol/g) was treated with ethylenediamine as described above. The derivatized resin was used for synthesis according to "continuous-flow technique". 10 eq. (+/-)-4-methoxymandelic acid, 10 eq. HObt and 10 eq. DIC dissolved in 5 ml DMF, preactivated for 10 min was introduced at the top of the column and the synthesizer was operated in recirculation mode for 2 h. Excess of reagent was then removed by DMF washing (12 min, flow rate 1 ml/min). A small resin-sample was removed in order to check the acylation by the ninhydrin test. The first amino acid according to the sequence was coupled as Fmoc protected and side chain protected preformed symmetrical anhydride as described above and introduced at the top of the column together with (0.1 eq.) DMAP and the synthesizer was operated in recirculation mode for 90 min. Excess reagent was then removed by DMF washing (12 min, flow rate 1 ml/min). A small resin-sample was removed in order to check the coupling yield, which was estimated as described above and found to be 75%. The synthesis was then continued by cleavage of the Fmoc group as ear-

lier described. The remaining amino acids according to the sequence were coupled as Fmoc-protected Pfp esters (3 eq.) with the addition of Dhbt-OH (1 eq.). The end-point of each coupling was determined automatically as described above. After completed synthesis the peptide-resin was washed with DMF (10 min, flow rate 1 ml/min), DCM (3x5 ml, 1 min each)and finally diethyl ether, removed from the column and dried *in vacuo*.

The peptide was cleaved from the resin as earlier described and lyophilized from acetic acid-water.

The crude freeze dried product was analyzed by hplc and found to comprise the target peptide H-Ala$_n$-Lys-OH (n = 10) as well as deletion peptides corresponding to n = 9, 8, 7 and 6. The identity of the peptides were confirmed by MALDI TOF MS.

Example 12. Synthesis of H-Ala$_{10}$-Lys-OH using (Lys(Boc))$_6$ as pre-sequence and (+/-)-4-methoxymandelic acid as linker (H-Ala$_{10}$-Lys(Boc)-OCH-(4-MeOPh)CO-(Lys(Boc))$_6$-NHCH$_2$CH$_2$NH PepSyn K resin)

500 mg dry PepSyn K (0.1 mmol/g) was treated with ethylenediamine as described above. The derivatized resin was used for synthesis according to "continuous-flow technique". The first 6 Lysines forming the pre-sequence were coupled as Fmoc-protected and side chain protected Pfp esters (3 eq.) with the addition of Dhbt-OH (1 eq.). The end-point of each coupling was determined automatically as described above. The Fmoc group was cleaved as described above. After completed synthesis of the pre-sequence 10 eq. (+/-)-4-methoxymandelic acid was coupled as preactivated HObt-ester as earlier described and introduced at the top of the column. The

synthesizer was operated in recirculation mode for 2 h and excess reagent was then removed by DMF washing (12 min, flow rate 1 ml/min). A small resin-sample was removed in order to check the acylation by the ninhydrin test. The next amino acid according to the sequence was coupled as Fmoc protected and side chain protected preformed symmetrical anhydride as earlier described and introduced at the top of the column together with (0.1 eq.) DMAP. The synthesizer was operated in recirculation mode for 90 min and excess reagent was then removed by DMF washing (12 min, flow rate 1 ml/min). A small resin-sample was removed in order to check the coupling yield, which was estimated as described above and found to be 68%. The synthesis was then continued by cleavage of the Fmoc group as earlier described. The remaining amino acids according to the sequence were coupled as Fmoc-protected Pfp esters (3 eq.) with the addition of Dhbt-OH (1 eq.). The end-point of each coupling was determined automatically as described above. After completed synthesis the peptide-resin was washed with DMF (10 min, flow rate 1 ml/min), DCM (3x5 ml min, flow rate 1 ml/min) and finally diethyl ether (3x5 ml min, flow rate 1 ml/min), removed from the column and dried *in vacuo*. The peptide was cleaved from the resin as earlier described and freeze dried from acetic acid-water. The crude freeze dried product was analyzed by hplc and found to comprise the target peptide H-Ala$_n$-Lys-OH (n = 10) as well as deletion peptides corresponding to n = 9, 8, 7 and 6. The amount of deletion peptides was found to be significantly reduced compared to examples 10 and 11 (see fig. 10). Fmoc-protected sequences were not detected. The identity of the peptides were confirmed by MALDI TOF MS.

Example 13. Synthesis of H-Ala$_{10}$-Lys-OH using (Lys(Boc))$_6$ as pre-sequence and (+)-4-methoxymandelic acid as linker (H-Ala$_{10}$-Lys(Boc)-OCH-(4-MeOPh)CO-(Lys(Boc))$_6$-NHCH$_2$CH$_2$NH PepSyn K resin) .

Dry PepSyn K (ca 500 mg, 0.1 mmol/g) was placed in a polyethylene vessel equipped with a polypropylene filter for filtration and treated with ethylenediamine as earlier described. The first 6 Lysines forming the pre-sequence were coupled as Fmoc-protected and side chain protected Pfp esters (3 eq.) with the addition of Dhbt-OH (1 eq.). The acylations were checked by the ninhydrin test performed at 80°C as described above. The Fmoc group was deprotected as described above. After finishing the pre-sequence the deprotected peptide-resin was reacted with 10 eq. (+)-4-methoxymandelic acid as a preactivated HObt-ester as described above (resolved as described above, 95.8% optical purity)and the coupling was continued for 24 h. Excess reagent was removed by DMF washing (12 min flow rate 1 ml/min). The acylation was checked by the ninhydrin test. The next amino acid according to the sequence was coupled as Fmoc protected and side chain protected preformed symmetrical anhydride as described above and the reaction was continued for 2 h. Excess reagent was then removed by DMF washing (12 min flow rate 1 ml/min). A small resin-sample was removed in order to check the coupling yield, which was estimated as described above, and found to be 66%. The synthesis was then continued by cleavage of the Fmoc group as described above.

The first alanine was coupled as a Fmoc-protected Pfp ester (3 eq.) with the addition of Dhbt-OH (1 eq.) in DMF (2 ml) for 2 h. Excess reagent was then removed

by DMF washing (12 min flow rate 1 ml/min) and the acylation was checked by the ninhydrin test performed at 80 °C as described above. The Fmoc group was then removed by treatment with 2% piperidine in DMF v/v (1 min, flow rate 1 ml/min) followed by flushing with DMF (10 sec, flow rate 10 ml/min), flowing with 0.2% Dhbt-OH in DMF (20 min, flow rate 1 ml/min) and finally washing with DMF (2x5 ml, 1 min each). The following Fmoc-protected alanine was coupled immediately as a Pfp ester (3 eq.) with the addition of 1 eq Dhbt-OH in DMF (2 ml) for 2 h. The acylation was checked by the ninhydrin test performed as described above. The remaining amino acids according to the sequence were coupled as Fmoc-protected Pfp esters (3 eq.) with the addition of Dhbt-OH (1 eq.) in DMF (2 ml). Excess reagent was removed by DMF washing (12 min flow rate 1 ml/min) and acylations were checked by the ninhydrin test performed 80 °C as described above. The Fmoc group was deprotected as described above. After completed synthesis the peptide-resin was washed with DMF (10 min, flow rate 1 ml/min), DCM (3x5 ml, 1 min each), diethyl ether (3x5 ml, 1 min each) and dried *in vacuo*.

The peptide was cleaved from the resin as described above and freeze dried from glacial acetic acid. The crude freeze dried product was analyzed by hplc and found to be homogeneous without deletion and Fmoc-protected sequences.

Example 14. Synthesis of H-Ala$_{10}$-Lys-OH using (Glu(OtBu))$_6$ as pre-sequence and (+)-4-methoxymandelic acid as linker (H-Ala$_{10}$-Lys(Boc)-OCH-(4-MeOPh)CO-(Glu(OtBu))$_6$-NHCH$_2$CH$_2$NH PepSyn K resin).

Dry PepSyn K (ca 500 mg) was placed in a polyethylene vessel equipped with a polypropylene filter for filtration and treated with ethylenediamine as described above. The first 6 glutamic acids forming the pre-sequence were coupled as Fmoc-Glu(OtBu)-Pfp esters (3 eq.) with the addition of Dhbt-OH (1 eq.). The acylations were checked by the ninhydrin test performed at 80°C as described above. The Fmoc group was deprotected as described above. After finishing the pre-sequence 10 eq. (+)-4-methoxymandelic acid (resolved as described above, 95.8% optical purity) was coupled as a preactivated HObt-ester as described above. The coupling was continued for 24 h and excess reagent was then removed by DMF washing (12 min flow rate 1 ml/min). The acylation was checked by the ninhydrin test. The next amino acid according to the sequence was coupled as Fmoc protected and side chain protected preformed symmetrical anhydride as described above catalyzed by DMAP (0.1 eq.) and the reaction was continued for 2 h. Excess reagent was removed by DMF washing (12 min flow rate 1 ml/min). A small resin-sample was removed in order to check the coupling yield, which was estimated as described above and the yield found to be 75%. The synthesis was then continued by cleavage of the Fmoc group as described above.

The first alanine was coupled as a Fmoc-protected Pfp ester (3 eq.) with the addition of Dhbt-OH (1 eq.) in DMF (2 ml) for 2 h. Excess reagent was then removed by DMF washing (12 min flow rate 1 ml/min) and the

acylation was checked by the ninhydrin test performed at 80 °C as described above. The Fmoc group was then removed by treatment with 2% piperidine in DMF v/v (1 min, flow rate 1 ml/min) followed by flushing with DMF (10 sec, flow rate 10 ml/min), flowing with 0.2% Dhbt-OH in DMF (20 min, flow rate 1 ml/min) and finally washing with DMF (2x5 ml, 1 min each). The following Fmoc-protected alanine was coupled immediately as a Pfp ester (3 eq.) with the addition of 1 eq Dhbt-OH in DMF (2 ml) for 2 h. The acylation was checked by the ninhydrin test performed as described above. The remaining amino acids according to the sequence were coupled as Fmoc-protected Pfp esters (3 eq.) with the addition of Dhbt-OH (1 eq.) in DMF (2 ml). Excess reagent was removed by DMF washing (12 min flow rate 1 ml/min) and acylations were checked by the ninhydrin test performed 80 °C as described above. The Fmoc group was deprotected as described above. After completed synthesis the peptide-resin was washed with DMF (10 min, flow rate 1 ml/min), DCM (3x5 ml, 1 min each), diethyl ether (3x5 ml, 1 min each) and dried *in vacuo*.

The peptide was cleaved from the resin as described above and freeze dried from glacial acetic acid. The crude freeze dried product was analyzed by hplc and found to be homogeneous without deletion and Fmoc-protected sequences.

Example 15. Peptide synthesis of Tyr-Gly-Gly-Phe-Leu-Lys_6-OH on NovaSyn TentaGel.

Dry NovaSyn TG resin (0.29 mmol/g, 250 mg) was placed in a polyethylene vessel equipped with a polypropylene filter for filtration and treated as described under "batchwise peptide synthesis on PEG-PS"

until finishing the pre-sequence Lys_6. The following amino acids forming the Leu-enkephaline sequence were coupled as preformed Fmoc-protected HObt esters (3 eq.) in DMF (5 ml) generated by means of DIC. Before each of the last five couplings the resin was washed with a solution of Dhbt-OH (80 mg in 25 ml), in order to follow the disappearance of the yellow color as the coupling reaction proceed. When the yellow color was no longer visible the couplings were interrupted by washing the resin with DMF (5 x 5 ml, 5 min each). The acylations were then checked by the ninhydrin test performed at 80 °C as earlier described. After completed synthesis the peptide-resin was washed with DMF (3x5 ml, 1 min each), DCM (3x5 ml, 1 min each), diethyl ether (3x5 ml, 1 min each) and dried *in vacuo*.

The peptide was cleaved from the resin as described above and freeze dried from acetic acid. The crude freeze dried product was analyzed by hplc and found to be homogeneous without deletion and Fmoc-protected sequences. The purity was found to be better than 98% and the identity of the peptide was confirmed by ES-MS. Yield 84%.

Table 1.

Amino acid	Peptide-resin	Coupling time	
	Pre-sequence: (Lys(tBoc))6	With pre-sq.	Without pre-sq.
Fmoc-Leu-OH	H-Lys(Boc)6-HMPA-R	< 2 min	-
Fmoc-Phe-OH	H-Leu-Lys(Boc)6-HMPA-R	< 5 min	< 120 min
Fmoc-Gly-OH	H-Phe-Leu-Lys(Boc)6-HMPA-R	< 2 min	< 60 min
Fmoc-Gly-OH	H-Gly-Phe-Leu-Lys(tBoc)6-HMPA-R	< 2 min	< 60 min
Fmoc-Tyr-OH	H-Gly-Gly-Phe-Leu-Lys(tBoc)6-HMPA-R	< 1 min	< 40 min
	H-Tyr-Gly-Gly-Phe-Leu-Lys(tBoc)6-HMPA-R		

R= NovaSyn TG derivatized with the linker 4-hydroxymethyl-phenoxy acetic acid (HMPA) (Subst.: 0.29 mmol/g). Coupling times are based on the Dhbt-OH and the ninhydrin test ,

Materials and methods

Abbreviations:

AM, p-[(R,S)-a[1-(9H-fluoren9-yl)-methoxyformamido]-2,4-dimethoxybenzyl]-phenoxyacetic acid
At, 7-azabenzotriazol-1-yl
Boc, tert. butyloxycarbonyl
BOP, benzotriazolyl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate
Bpoc, biphenylpropyloxycarbonyl
Bt, benzotriazol-1-yl
tBu, tert. butyl
DBU, diazabicyclo[5,4,0]undec-7-ene
Ddz, 3,5-dimethoxyphenylisopropyloxycarbonyl
DCC, dicyclohexylcarbodiimide
DCM, dichloromethane
DIC, diisopropylcarbodiimide
DIEA, N,N-diisopropylethylamine
DMAP, 4-(N,N-dimethylamino)pyridine
Dhbt-OH, 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine
DMF, N,N-dimethylformamide
Dts, dithiasuccinyl
EDT, ethanedithiol
FAB, fast atom bombardment
Fmoc, 9-fluorenylmethyloxycarbonyl
HATU, O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexaflurophosphate
HBTU, O-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HMPA, 4-hydroxymethylphenoxyacetic acid
HMPB, 4-(4-hydroxymethyl-3-methoxyphenoxy)-butyric acid
HObt, 1-hydroxybenzotriazole
HOAt, 1-hydroxy-7-azobenzotriazole

HPLC, high pressure/liquid chromatography
MCPS, multiple column peptide synthesis
MHC, major histocompatibility complex
MMa, 4-methoxymandelic acid
NMR, nuclear magnetic resonance
NHS, N-hydroxy-succinic-acid imido ester
NMP, N-methylpyrrolidone
NPS, nitrophenylsulfenyl
Mtr, 4-methoxy-2,3,6-trimethylphenylsulfonyl
PAM, phenylacetamidomethyl
Pbf, 2,2,4,6,7-pentamehtyldihydrobenzofuran-5-sulfonyl
PEG-PS, polyethyleneglycol grafted on polystyrene
PepSyn Gel, polydimethylacrylamide resin functionalized with sarcosine methylester
PepSyn K, Kieselguhr supported polydimethylacrylamide resin functionalized with sarcosine methylester
Pfp, pentafluorophenyl
Pmc, 2,2,5,7,8-pentamethylchorman-6-sulfonyl
Poc, phenylisopropyloxycarbonyl
TBTU, O-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate
TFA, trifluoroacetic acid
TFE, trifluorethanol
TFMSA, trifluoromethanesulfonic acid
Tmz, 2,4,5-tetramethylbenzyloxycarboxyl

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P A T E N T C L A I M S

1. A process for the production of peptides

$X\text{-}AA_1\text{-}AA_2$ .....$AA_n\text{-}Y$

wherein AA is an L- or D-amino acid residue,

X is hydrogen or an amino protective group

Y is OH, NH_2 or an amino acid sequence comprising from 3 to 9 amino acid residues and n is an integer greater than 2 by solid phase synthesis wherein the C-terminal amino acid in the form of an N-α-protected, if necessary side chain protected reactive derivative is coupled to a solid support or a polymer optionally by means of a linker, subsequently N-α-deprotected, whereafter the subsequent amino acids forming the peptide sequence are stepwise coupled or coupled as a peptide fragment in the form of suitably protected reactive derivatives or fragments, wherein the N-α-protective group is removed following formation of the desired peptide and the peptide is cleaved from the solid support,

c h a r a c t e r i z e d in that the C-terminal part attached to the support or polymer comprises a pre-sequence comprising from 3 to 9, preferably from 5 to 7 amino acid residues independently selected from native L-amino acids having a side chain functionality which is protected during the coupling steps and having a propensity factor $P\alpha > 0,57$ and a propensity factor $P\beta > 1,10$ or the corresponding D-amino acids and said pre-sequence is optionally cleaved from the formed peptide.

2. The process according to claim 1, wherein the amino acids in the pre-sequence are chosen from amino

acids having a side chain functionality which is a carboxy, carboxamido, amino, hydroxy, guanidino, sulphide or imidazole group

3. The process according to claim 2, wherein the amino acids forming part of the pre-sequence are independently selected from Lys, Glu, Asp, Ser, His, Asn, Arg, Met and Gln.

4. The process according to claim 3, wherein the amino acids are either exclusively Lys or Glu or a sequence (Glu)q(Lys)p, where p + q is 3 to 9, preferably 6 to 9, and the order of Lys and Glu is arbitrarily chosen.

5. The process according to claim 1, wherein the N-α amino protective group is Fmoc, Boc or any other suitable protective group.

6. The process according to claim 5, wherein the N-α-amino protective group is Fmoc or Boc.

7. The process according to claim 1, wherein the side chain functionality in the pre-sequence comprises a carboxy group, which is suitably protected, preferably with tBu.

8. The process according to claim 1, wherein the side chain functionality in the pre-sequence comprises an amino group, which is suitably protected, preferably with Boc.

9. The process according to claim 1, wherein the side chain functionality in the pre-sequence comprises a hydroxy group, which is suitably protected preferably with tBu.

10. The process according to claim 1, wherein the side chain functionality in the pre-sequence comprises a carboxamido group protected with a) benzhydryl, b) trityl, c) tBu.

11. The process according to claim 1, wherein the side chain functionality in the pre-sequence comprises a guanidino group protected with 4-methoxy-2,3,6-trimethylphenylsulfonyl (Mtr), 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) or 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf).

12. The process according to claim 1, wherein the side chain functionality in the pre-sequence comprises an imidazole group protected with Boc.

13. The process according to claim 5, wherein the Fmoc groups are deprotected by means of an amine such as piperidine or diazabicyclo[5,4,0]undec-7-ene (DBU).

14. The process according to claim 1, wherein the side chain protective groups are deprotected by means of an acid such as trifluoroacetic acid (TFA), trifluoromethanesulfonic acid (TFMSA), HBr, HCl or HF.

15. The process according to claim 1, wherein the solid support is selected from functionalized resins such as polystyrene, polyacrylamide, polyethyleneglycol, cellulose, polyethylene, latex or dynabeads.

16. The process according to claim 15, wherein the C-terminal amino acid is attached to the solid support by means of a common linker such as 2,4-dimethoxy-4'-hydroxy-benzophenone, 4-(4-hydroxymethyl-3-methoxyphenoxy)-butyric acid (HMPB), 4-hydroxy-

methylbenzoic acid, 4-hydroxymethylphenoxyacetic acid (HMPA), 3-(4-hydroxymethylphenoxy)propionic acid or p-[(R,S)-a[1-(9H-fluoren-9-yl)-methoxyformamido]-2,4-dimethoxybenzyl]-phenoxyacetic acid (AM).

17. The process according to claim 1, wherein the synthesis is carried out batchwise.

18. The process according to claim 1, wherein the process is carried out continuously on an automated or semi automated peptide synthesizer.

19. The process according to claim 1, wherein the coupling steps are performed in the presence of a solvent selected from acetonitrile, N,N-dimethylformamide (DMF), N-methylpyrrolidone (NMP), dichlormethane (DCM), trifluoroethanol (TFE), ethanol, methanol, water, mixtures of the mentioned solvents with or without additives such as perchlorate or ethylenecarbonate.

20. The process according to claim 1, wherein the coupling between two amino acids, an amino acid and the earlier formed peptide sequence or a peptide fragment and the earlier formed peptide sequence is carried out according to usual condensation methods such as the azide method, mixed acid anhydride method, symmetrical anhydride method, carbodiimide method, active ester method such as pentafluorophenyl(Pfp), 3,4-dihydro-4-oxobenzotriazin-3-yl (Dhbt), benzotriazol-1-yl (HOBt), 7-azabenzotriazol-1-yl (HOAt), 4-nitrophenyl, N-hydroxysuccinic acid imido esters (NHS), acid chlorides, acid fluorides, in situ activation by O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU), O-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), O-(benzotriazol-

1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU), or benzotriazolyl-oxy-tris-(dimethylamio)-phosphonium hexafluorophosphate (BOP).

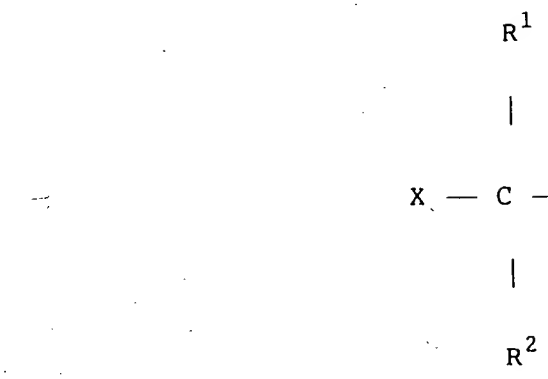
21. The process according to claim 1, wherein the peptide is cleaved from the support by means of an acid such as trifluoroacetic acid (TFA), trifluoromethanesulfonic acid (TFMSA), hydrogen bromide (HBr), hydrogen chloride (HCl), hydrogen fluoride (HF) or a base such as ammonia, hydrazine, an alkoxide or a hydroxide.

22. The process according to claim 1, wherein the peptide is cleaved from the support by means of photolysis.

23. The process according to claim 1, characterized by inserting a linker between the pre-sequence attached to a support and the AA_1-AA_n sequence which enables a selective cleavage of said sequence.

24. The process according to claim 23, wherein the linker is optically active.

25. The process according to claim 24, wherein the linker is an α-hydroxy or α-amino acid of the general formula



wherein X is OH or NH_2, and R^1 and R^2 are independently selected from H, C_{1-3} alkyl, phenyl and substituted phenyl, where the substitutents are one or more electron donating substituents chosen among C_{1-3} alkoxy, C_{1-3} alkyl, or two vicinal substituent groups are joined to form a 5 or 6 membered carbon ring together with the carbon atoms to which they are attached.

26. The process according to claim 25, wherein the linker is (+)-4-methoxymandelic acid, diphenylglycin or glycolic acid.

27. The process according to 23, wherein the AA_1-AA_n sequence is cleaved from the pre-sequence by means of an acid such as trifluoroacetic acid (TFA), trifluoromethanesulfonic acid (TFMSA), hydrogen bromide (HBr), hydrogen chloride (HCl), hydrogen fluoride (HF) or a base such as ammonia, hydrazine, an alkoxide or a hydroxide.

28. The process according to claim 23, wherein the pre-sequence is enzymatically cleaved from the formed peptide.

29. The process according to claim 28, wherein the enzyme is selected from suitable carboxy- and endopeptidases.

30. The process according to claim 23, characterized by inserting a first linker between the pre-sequence attached to a support and the AA_1-AA_n sequence and a second linker between the pre-sequence and the solid support with orthogonal cleavage conditions to the first linker enabling a selective cleavage of the second linker by means of trifluoroacetic acid (TFA), trifluoromethanesulfonic acid (TFMSA), HBr, HCl, HF or a base such as ammonia, hydrazine, an alkoxide or a hydroxide to give a peptide AA_1-AA_n linked to the pre-sequence by means of said first linker.

31. An agent for use in solid phase peptide synthesis having the general formula

$X\text{-}AA'_1\text{-}...\text{-}AA'_m\text{-}Y_1\text{-}R$

wherein R is a solid support applicable in solid phase peptide synthesis, Y_1 is an amino acid sequence comprising from 3 to 9, preferably from 5 to 7 amino acid residues independently selected from L-amino acids having a side chain functionality which is protected during the coupling steps and having a propensity factor $P\alpha > 0,57$ and a propensity factor $P\beta \leq 1,10$, or the corresponding D-amino acid, AA' is an L- or D-amino acid residue, m is zero or an integer from 1 to 40 and X is hydrogen or an amino protective group

32. The agent according to claim 31, wherein the amino acids forming part of the Y_1 sequence are independently selected from Lys, Glu, Asp, Ser, His, Asn, Arg, Met and Gln.

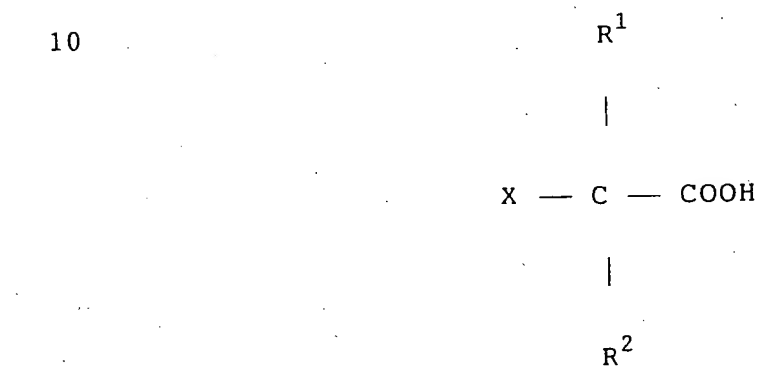
33. An agent for use in solid phase peptide synthesis having the general formula

$X\text{-}AA'_1\text{-}...\text{-}AA'_m\text{-}L_1\text{-}Y_1\text{-}R$

wherein X, AA', m, Y_1 and R are as defined in claim 31 and L_1 is a linker which enables a selective cleavage of the bond to AA'_m.

34. The agent according to claim 33, wherein the linker L_1 is optically active.

35. The agent according to claim 33, wherein the linker L_1 is an α-hydroxy or α-amino acid of the general formula



wherein X is OH or NH_2, and R^1 and R^2 are independently selected from H, C_{1-3} alkyl, phenyl and substituted phenyl, where the substitutents are one or more electron donating substituents chosen among C_{1-3} alkoxy, C_{1-3} alkyl, or two vicinal substituent groups are joined to form a 5 or 6 membered carbon ring together with the carbon atoms to which they are attached.

36. The agent according to claim 35, wherein the linker is 4-methoxymandelic acid, (+)-4-methoxymandelic acid, diphenylglycin or glycolic acid.

37. An agent for use in solid phase peptide synthesis having the general formula

$X\text{-}AA'_1\text{-}...\text{-}AA'_m\text{-}L_1\text{-}Y_1\text{-}L_2\text{-}R$

wherein X, AA', m, Y_1, R and L_1 are as defined in claim 33 and L_2 is a linker with orthogonal cleavage conditions to the first linker and enabling a selective cleavage from the solid support.

38. An agent for use in solid phase peptide synthesis having the general formula

$X\text{-}AA'_1\text{-}...\text{-}AA'_m\text{-}Y_1\text{-}L_2\text{-}R$

wherein X, AA', m, Y_1, R and L_2 is as defined in claim 37.

39. An agent according to claim 38, wherein L_2 is selected from 2,4-dimethoxy-4'-hydroxy-benzophenone, 4-(4-hydroxymethyl-3-methoxyphenoxy)-butyric acid (HMPB), 4-hydroxymethylbenzoic acid, 4-hydroxymethylphenoxyacetic acid (HMPA), 3-(4-hydroxymethylphenoxy)propionic acid or p-[(R,S)-a[1-(9H-fluoren-9-yl)-methoxyformamido]-2,4-dimethoxybenzyl]-phenoxyacetic acid (AM).

Fig. 1

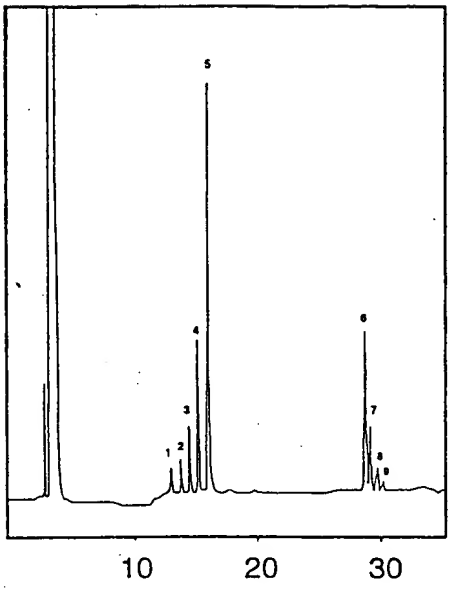


Fig. 2

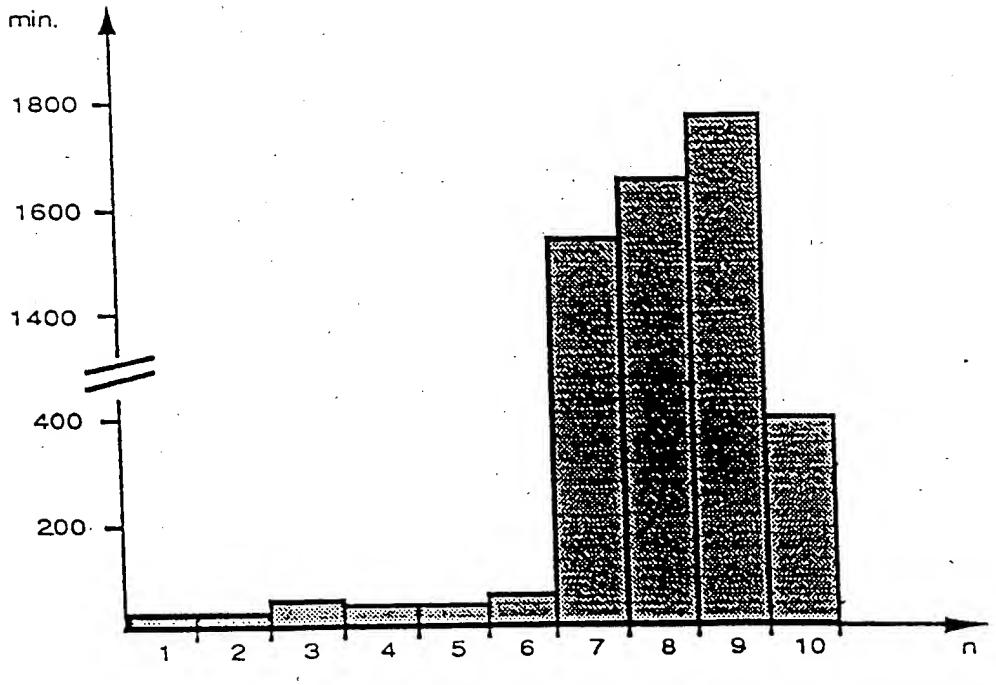


Fig. 3

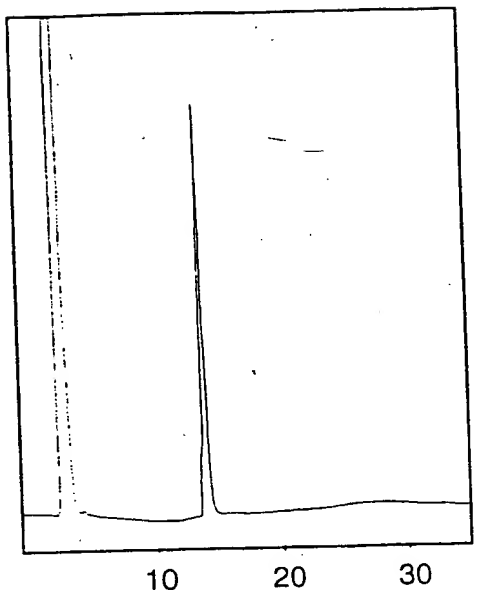


Fig. 4

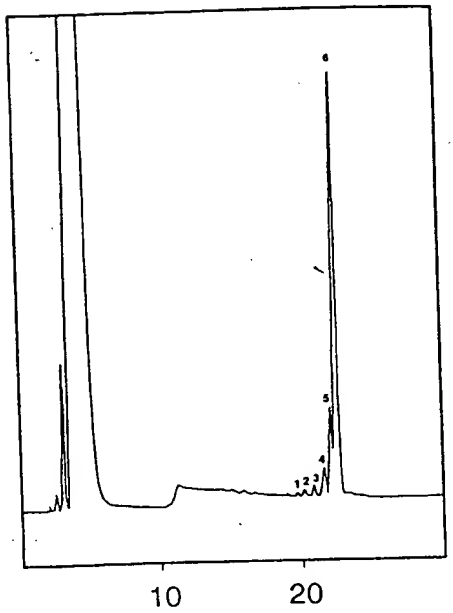


Fig. 5

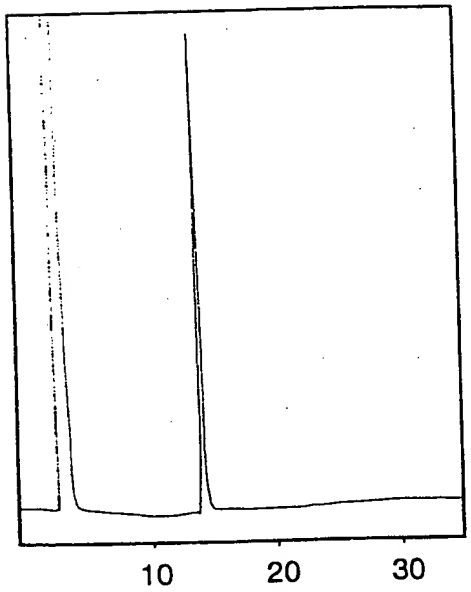


Fig. 6

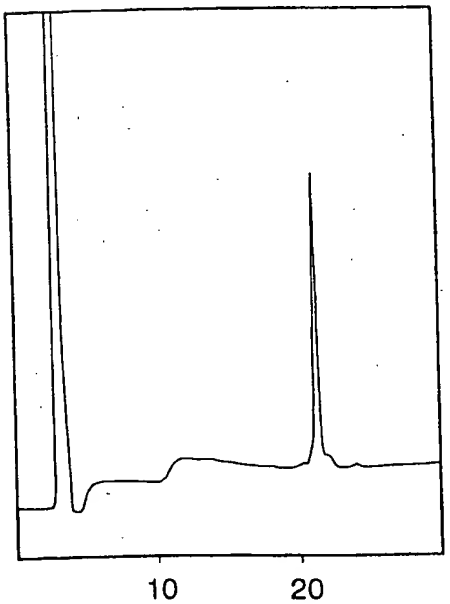


Fig. 7

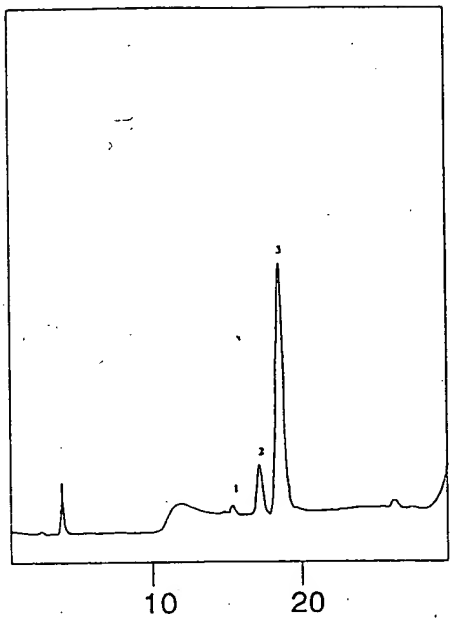


Fig. 8

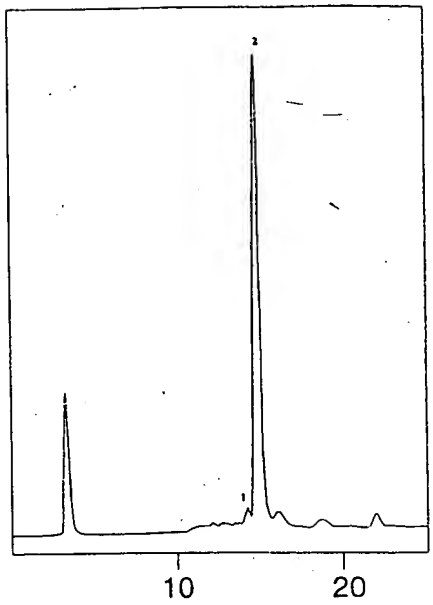


Fig. 9

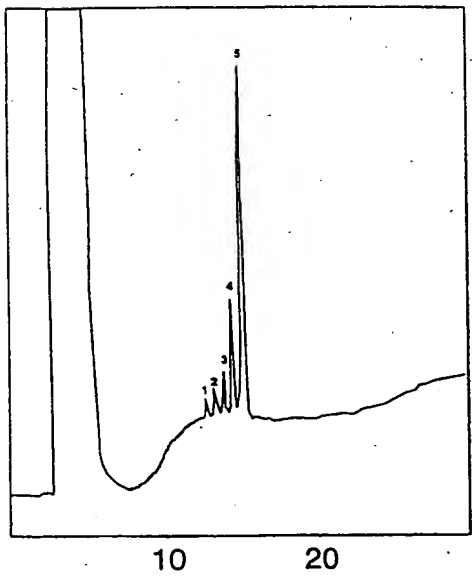
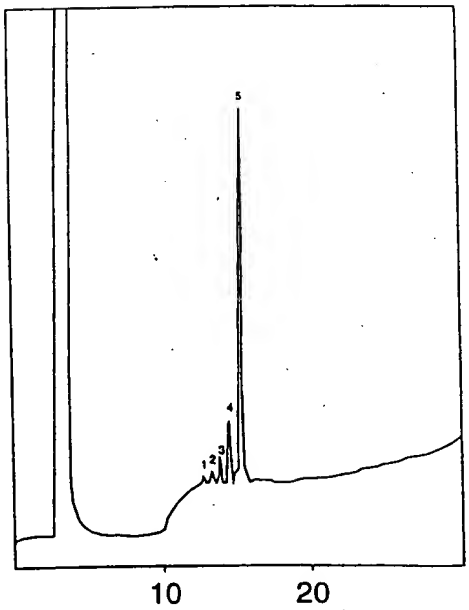


Fig. 10



INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 97/00375

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C07K 1/04
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, CA, US PATENTS FULLTEXT, MEDLINE, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5021550 A (ALLEN R. ZEIGER), 4 June 1991 (04.06.91) -- --------	1-39

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 November 1997	01-12-1997
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Carolina Palmcrantz Telephone No. +46 8 782 25 00

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INTERNATIONAL SEARCH REPORT

Information on patent family members

01/10/97

International application No.
PCT/DK 97/00375

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5021550 A	04/06/91	US 5310894 A	10/05/94